

AD-A265 592 ENTATION PAGE

Form Approved
OMB No. 0704-0188

This report contains information which is being furnished to you by the Department of Defense under a contract with the National Technical Information Service. It is the property of the Department of Defense and is loaned to you. It and its contents are not to be distributed outside your agency. If you are not an agency of the Department of Defense, you are to destroy this report when it is no longer needed for your agency's use. If you are an agency of the Department of Defense, you are to destroy this report when it is no longer needed for your agency's use. If you are a contractor or subcontractor of the Department of Defense, you are to destroy this report when it is no longer needed for your agency's use. If you are a contractor or subcontractor of the Department of Defense, you are to destroy this report when it is no longer needed for your agency's use.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 1988		3. REPORT TYPE AND DATES COVERED Reprint	
4. TITLE AND SUBTITLE Purification and Characterization of a Family of High Molecular Weight Surface-Array Proteins from Campylobacter fetus				5. FUNDING NUMBERS 86PP6826 61102A 30161102BS13 AB DA312588	
6. AUTHOR(S) Zhiheng Pei, Richard T. Ellison III, Randolph V. Lewis, and Martin J. Blaser					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Veterans Administration Medical Center 1055 Clermont Street Denver, Colorado 80220				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research & Development Command Fort Detrick Frederick, MD 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Contract Title: Studies of the Outer Membrane Proteins of Campylobacter Jejuni for Vaccine Development					
12a. DISTRIBUTION AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE Accession For NTIS CRA&I <input checked="" type="checkbox"/> DTIC TAB <input checked="" type="checkbox"/> Unannounced <input type="checkbox"/> Justification	
13. ABSTRACT (Maximum 200 words) <div style="text-align: center;">DTIC ELECTE S D E MAY 28 1993</div> <div style="text-align: center;">DEF QUALITY INSPECTED 2</div> <div style="text-align: center;">93 6 02 01 6</div>				By _____ Distribution /	
				Availability Codes	
				Dist	Avail and/or Special
				A-1	20
14. SUBJECT TERMS				15. NUMBER OF PAGES	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified				18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	
19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified				20. LIMITATION OF ABSTRACT Unlimited	

Purification and Characterization of a Family of High Molecular Weight Surface-Array Proteins from *Campylobacter fetus**

(Received for publication, November 10, 1987)

Zhiheng Pei†, Richard T. Ellison III‡§, Randolph V. Lewis¶, and Martin J. Blaser†§**

From the †Medical and Research Services, Veterans Administration Medical Center and §Division of Infectious Diseases, Department of Medicine, University of Colorado School of Medicine, Denver, Colorado 80220 and the ¶Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071

A variety of Gram-negative and Gram-positive bacteria possess crystalline surface layers, although little is known of their function. We previously have shown that the high molecular weight surface-array proteins of *Campylobacter fetus* are important in both the pathogenicity and antigenicity of this organism. For biochemical and immunological characterization, we purified high molecular weight (100,000, 127,000, 149,000) surface-array proteins from three *C. fetus* strains using sequential gel filtration and ion exchange high performance liquid chromatography. These proteins are acidic with pI values between 4.12 and 4.25 and contain large proportions of acidic amino acids (19.7%–22.0%) in addition to hydrophobic amino acids (37.3%–38.5%). They share a novel amino-terminal sequence through at least 19 residues. Carbohydrate analysis using periodic acid-Schiff staining and treatment with trifluoromethanesulfonic acid shows no evidence of glycosylation. Antiserum to a purified $M_r = 100,000$ protein from *C. fetus* 82-40 I.P. cross-reacts with three other purified *C. fetus* surface-array proteins by enzyme-linked immunosorbent assay with titers $>12,800$. We conclude that: 1) there is a family of surface-array proteins of *C. fetus* with common structural and antigenic characteristics; 2) that these molecules have similar biochemical characteristics to surface-array proteins described for other bacteria; but however, 3) by amino-terminal sequence analysis these are unique.

It is now recognized that a wide variety of bacteria may possess surface layers of regularly arranged protein subunits (S-proteins) (1). These surface-arrays are noncovalently attached to the outer membrane of Gram-negative organisms or to the peptidoglycan of Gram-positive organisms. The ultrastructural basis of these layers has been well defined (2), but biochemical analysis has been limited. The S-proteins of *Aeromonas salmonicida*, a fish pathogen, and *Azotobacter vinelandii*, a nitrogen-fixing soil bacterium have been best

characterized. The S-proteins of these bacteria have regular structures, are relatively hydrophobic, and are readily extracted from the cell envelopes (3, 4).

Campylobacter fetus subspecies *fetus* is an important veterinary pathogen causing abortion in cattle and sheep (5) and a human enteric pathogen that causes systemic infections in compromised hosts (6–8). Our previous work has demonstrated the presence of several high molecular weight surface proteins that are important virulence factors for these organisms (9). *C. fetus* strains encapsulated by these surface-array proteins (S-proteins) are usually resistant to normal human serum and to phagocytosis by polymorphonuclear leukocytes, whereas spontaneously appearing unencapsulated mutants are sensitive to these host defenses (9, 10). Prior studies of *C. fetus* S-protein by McCoy *et al.* (11, 12) using Coomassie Blue staining determined a $M_r = 98,000$ glycoprotein containing 3.8% carbohydrate which formed a hexagonal subunit capsular structure on the outer surface of the cell. The surface-exposure and pathogenic importance of the *C. fetus* S-proteins suggest that they will be potential candidates for a *C. fetus* vaccine. A vaccine prepared from bacteria-free high molecular weight protein-enriched supernatants of *C. fetus* broth culture protected pregnant ewes against challenge with live homologous *C. fetus* (13).

The present work is part of an investigation in the pathogenicity of *C. fetus* (14). Emphasis has been placed on developing a method to isolate and purify these S-proteins and to determine biochemical and immunological characteristics of S-proteins from different *C. fetus* strains.

EXPERIMENTAL PROCEDURES

Growth of Bacteria and Extraction of Surface Proteins—Five *C. fetus* strains (82-40 LP, 82-40 HP, 82-40 LP3, 84-86, and 84-112 from the culture collection of the Denver Veterans Administration Medical Center *Campylobacter* laboratory) were used in this study. Strains 82-40 LP, 84-86, and 84-112 originally were isolated from ill humans or animals. These strains had been passaged 5 to 10 times on artificial media. Strain 82-40 HP is a spontaneous mutant of 82-40 LP that lacks the $M_r = 100,000$ S-protein (9), and 82-40 LP3 is a spontaneous mutant in which the predominant form of the S-protein is a $M_r = 127,000$ structure. All strains were maintained frozen at -70°C in brucella broth (BBL Microbiology Systems, Cockeysville, MD) containing 15% glycerol for use as stocks. For studies, bacteria were grown on trypticase soy agar with 5% sheep blood (PASCO, Wheat Ridge, CO) in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, and 85% nitrogen) at 37°C for 48 h for the first two generations, and for 24 h for the third generation. Cells were harvested and washed five times with distilled water by centrifugation at $3,500 \times g$ for 15 min with the supernatants from each wash separately collected for subsequent comparisons. Acid-labile surface proteins were extracted with 0.2 M glycine hydrochloride buffer, pH 2.2, as previously described (10). Proteins were similarly extracted using 10 mM EDTA, 0.9% NaCl, or 100% hexadecane in place of the glycine

* This work was supported by Veterans Administration Research Service, Biomedical Research Grant BRSG-0537 from the National Institutes of Health, and National Institutes of Health Grant RR02035. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ National Institutes of Health Research Career Development Awardee and Sloan Foundation Research Fellow.

** Clinical Investigator of the Veterans Administration. To whom reprint requests should be addressed: Infectious Disease Section, Veterans Administration Medical Center, 1055 Clermont Street, Denver, CO 80220.

hydrochloride buffer and using 5 mM calcium chloride in place of water in this procedure.

Analytical Procedures—Protein concentration was measured by using the Markwell *et al.* modification of the Lowry method (15). SDS-PAGE¹ was performed in a modified Laemmli gel system as described by Ames (16). Proteins were resolved using the modified silver stain of Oakley *et al.* (17). Proteins were stained for carbohydrate content with periodic acid-Schiff's reagent as described previously (18). Molecular weight standards (Bio-Rad) were assigned as follows: myosin (200,000), β -galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,000). Determination of the pI of the S-proteins was made by isoelectric focusing in Resolve² thin layer agarose gels with pH range between 3 and 7 using the Resolve-Hb kit (Isolab Inc., Akron, OH) and the modified silver stain of Willoughby and Lambert (19). Isoelectric focusing standards (Sigma) were amyloglucosidase (3.55), trypsin inhibitor (4.55), and β -lactoglobulin A (5.13).

High Performance Liquid Chromatographic Procedures—Gel filtration HPLC was performed on a Superose 12 column (Pharmacia LKB Biotechnology Inc.) with a pH 7.5, 20 mM Tris, 50 mM NaCl at a flow rate of 0.15 ml/min. Ion exchange HPLC was performed on a Mono Q column (Pharmacia LKB Biotechnology Inc.) with a pH 7.5, 20 mM Tris buffer for the $M_r = 100,000$ protein from 82-40 LP and with a pH 5.0, 20 mM piperazine buffer for all other S-proteins at a flow rate of 0.5 ml/min and a linear gradient to 350 mM NaCl over 40 min. For all of these analyses, the column eluates were monitored for UV absorbance at 280 nm.

TFMS Deglycosylation—Deglycosylation with TFMS was performed by the method of Stewart *et al.* (20). Briefly, 1 ml of anisole and 2 ml of TFMS were mixed and cooled at 0 °C; 50 μ g of lyophilized protein was dissolved in 40 μ l of this mixture, and nitrogen was bubbled through the solution for 30 s. The mixture was agitated at 0 °C for 2 h, and the reaction was terminated by the addition of 250 μ l of pyridine/water (4:1, v/v). The solution was dialyzed against water for 18 h at 4 °C and then lyophilized.

Amino Acid Analysis and Sequencing—Purified S-proteins were prepared for analysis by dialysis against water and lyophilization. Amino acid analysis was performed using the method of Jones (21). The purified proteins (400–500 pmol) were sequenced on an Applied Biosystems 470 A Protein Sequencer equipped with the 120A Autoanalyzer using the O3RPTH program. The column for the separation of the phenylthiohydantoin-amino acid derivatives was the phenylthiohydantoin C-18 (220 \times 2.1 mm, 5 μ m) with a gradient of: 0 min – 8% B, 120 μ l/min; 0.1 min – flow to 200 μ l/min; 11 min – 12%; 30 min – 30% B, flow to 230 μ l/min; 37 min – 36% B; 37.1 min – 80% B; 40 min – flow to 300 μ l/min; 48 min – 80% B. The buffers were: A, 5% tetrahydrofuran in H₂O with 28.8 ml/liter of 3 M sodium acetate, pH 3.8, and 6.7 ml/liter of 3 M sodium acetate, pH 4.6; and B, acetonitrile. The column was at 55 °C. Comparison of the amino-terminal sequences with known proteins was performed by an on line search of the National Biomedical Research Foundation database (Georgetown, Washington, D.C.).

Immunological Methods—Antiserum against the *C. fetus* 82-40 LP $M_r = 100,000$ S-protein was raised in adult New Zealand White female rabbits by three subcutaneous injections at 2-week intervals of 40 μ g of purified protein in 1 ml of an equivolume mixture of antigen and adjuvant (67% 0.1 M PBS, 27% hexadecane, 6% glycerol). Fresh normal serum from the same rabbits was obtained prior to inoculation. Determination of antibody concentration and a comparison of the immunologic cross-reactivity of the purified S-proteins was performed by ELISA as previously described (22). To sensitize the ELISA plates (Immulon II; Dynatech Laboratories Inc., Alexandria, VA), purified proteins and the material from the second wash of 82-40 LP and 82-40 HP were diluted to 10 μ g/ml, and whole bacterial cells were diluted to 40 μ g/ml in 0.015 M carbonate buffer, pH 9.6. One hundred μ l of these preparations were added to each well, and the plates were incubated at 4 °C overnight. The plates were washed once with 0.01 M PBS, pH 7.2, in 0.03% Tween 20 and 0.01% thimerosal (PBS-T-T) and were blocked with 200 μ l/well of 0.1% gelatin in PBS-T-T overnight at 4 °C. The plates were washed twice

with PBS-T-T. 100 μ l of antisera was diluted with 0.5% bovine γ -globulin, and 0.1% gelatin in PBS-T-T was added in triplicate to each well. After a 1-h incubation at 37 °C, the plates were washed three times as before, 100 μ l of peroxidase goat anti-rabbit IgG (1:10,000, Boehringer Mannheim Biochemicals, San Diego, CA) was diluted with 0.1% bovine γ -globulin, and 1% BSA in PBS-T-T was added to each well and incubated at 37 °C for 1 h. The plates were washed five times, and 100 μ l of peroxidase developer (20 mg of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), 34 μ l of H₂O₂, 9.35 ml of 0.2 M Na₂HPO₄, and 10.65 ml of 0.1 M citric acid) was added to each well, and the product of the peroxidase reaction was determined after 30 min at room temperature using a MR 600 microplate reader (Dynatech Laboratories Inc., Alexandria, VA) at 414 nm. The titer was defined as the reciprocal of the highest dilution giving a reading of 0.1.

RESULTS

Water Extraction of Surface Proteins—The purification of the S-proteins from *C. fetus* involves extraction of the surface layer from whole cells followed by gel filtration and ion exchange chromatography. The first extraction of an overnight culture of *C. fetus* strain 82-40 LP with water yielded a protein content that was more than 6 times that of the second, third, fourth, and fifth washes combined, but primarily contained small molecular weight proteins from the blood agar media as determined by SDS-PAGE (Fig. 1). Analysis of the third, fourth, and fifth washes demonstrated an approximate 80% purification of the $M_r = 100,000$ S-protein, with approximately 0.1 mg of protein per plate in the third wash. Similar results were found in water extracts of *C. fetus* strains 84-86, 82-40 LP3 and 84-112. Optimal harvesting of the $M_r = 100,000$ S-protein of 82-40 LP was in the third wash, and of the $M_r = 100,000$ S-protein of 84-86 in the second wash. Optimal extraction of the $M_r = 127,000$ S-protein of 82-40 LP3 was in pooled second and third washes and of the $M_r = 149,000$ S-protein of 84-112 in the second wash. Water extraction contained more $M_r = 100,000$ S-protein and less contamination with small molecular weight proteins (Fig. 1) when compared with acid extraction or extraction with 0.9% NaCl, 10 mM EDTA, or hexadecane (data not shown), and contained 10 times more $M_r = 100,000$ S-protein than similar extraction with 5 mM calcium chloride. The $M_r = 100,000$ S-protein was stable during 20 min boiling in water (data not shown).

Protein Purification—The $M_r = 100,000$ S-protein of 82-40 LP was further purified by HPLC (see "Experimental Procedures"). The $M_r = 100,000$ S-protein eluted from the gel filtration column between 11.7 and 12.6 ml. The protein was further purified to homogeneity by ion exchange separation on a Mono Q column with the $M_r = 100,000$ S-protein eluting between 18.4% and 20.1% of buffer B.

The $M_r = 149,000$ S-protein from the second wash of 84-112, the $M_r = 100,000$ S-protein from 84-86, and the $M_r = 127,000$ S-protein from second wash and third wash of LP3 was purified by sequential ion exchange HPLC on Mono Q and gel filtration HPLC on Superose 12 (Fig. 2).

Isoelectric Focusing—An expedient formula of pI versus distance was generated by using three standard proteins of known pI between 3.55 and 5.13. All four S-proteins are acidic with pI values between 4.12 and 4.25 (Fig. 3). No relationship was found between molecular weight and pI.

Carbohydrate Analysis—To determine if any of the molecular weight differences of *C. fetus* S-protein was because of differential glycosylation, four *C. fetus* S-proteins were subjected to TFMS-mediated deglycosylation which removes O-linked and N-linked carbohydrates. After 120 min of incubation at 0 °C with TFMS, all four of the *C. fetus* S-proteins were decreased by about $M_r \sim 1000$. A similar decrease in the size of BSA (a nonglycosylated protein) was observed. In

¹ The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TFMS, trifluoromethanesulfonic acid; PBS, phosphate-buffered saline; PBS-T-T, thimerosal; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; EGTA, [ethylenedis(oxyethylenetriamino)]tetraacetic acid.

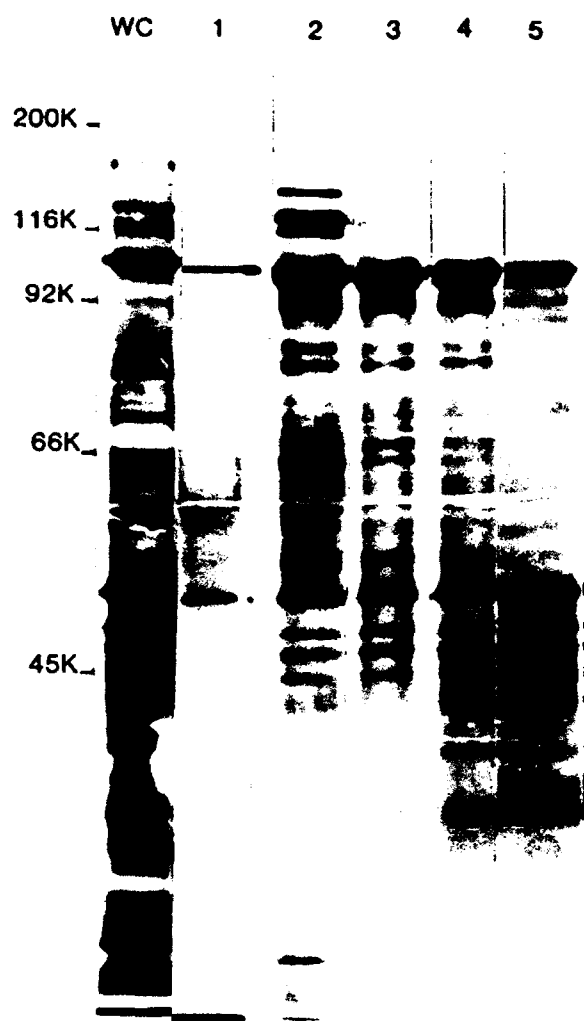


FIG. 1. SDS-PAGE of water extracts of *C. fetus* 82-40 low passage (LP). Lanes are from the whole cell preparation (WC), and after the first (1), second (2), third (3), fourth (4), and fifth (5) washes of whole cells in sterile distilled water. The amount of protein in these extracts is 29.1 mg (first), 2.1 mg (second), 1.4 mg (third), 0.6 mg (fourth), and 0.2 mg (fifth). In addition to the $M_r = 100,000$ S-protein, a second major S-protein in the second extraction migrated at $M_r = 127,000$.

contrast to the negative control of BSA and *C. fetus* S-proteins, TFMS treatment of fetuin caused a large decrease in the M_r of the major protein band. Partial degradation products in both control proteins and *C. fetus* S-proteins were observed. Because no larger changes were found in the size of *C. fetus* S-proteins than in that of negative control BSA, we conclude that S-proteins of *C. fetus* are glycosylated less than BSA, and the molecular weight differences of *C. fetus* S-proteins are not related to differential glycosylation. For confirmation, Schiff staining was performed with α_1 -acid glycoprotein (41% glycosylated) as a positive control and BSA as a negative control. The Schiff staining procedure could detect as low as $0.15 \mu\text{g}$ of carbohydrate per lane for α_1 -acid glycoprotein. No positive staining was found with $100 \mu\text{g/lane}$ of BSA or $100 \mu\text{g/lane}$ of four *C. fetus* S-proteins, indicating that glycosylation, if present, represented less than 0.15% of the molecular weight (data not shown).

Amino Acid Composition and Amino-terminal Amino Acid Sequences—The amino acid compositions of these four S-proteins were very similar to one another (Table I) and to

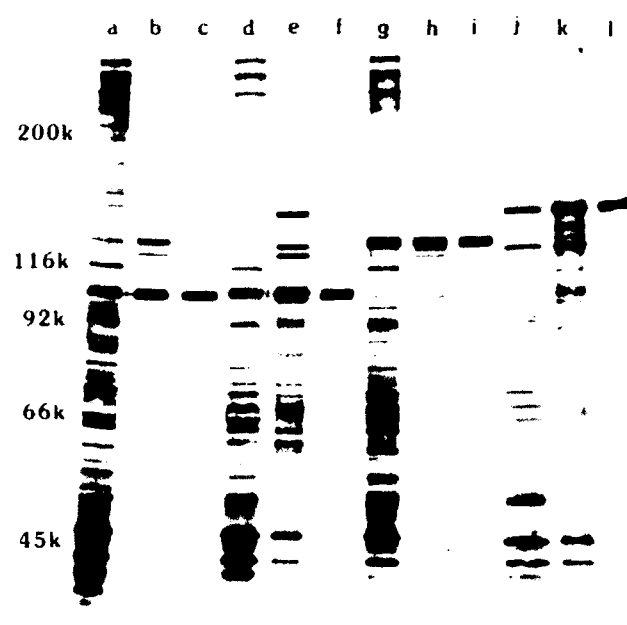


FIG. 2. Purification of *C. fetus* high molecular weight S-proteins. SDS-PAGE with 7% acrylamide. Lanes are: a, whole cell preparation of 82-40 LP; b, third water wash from 82-40 LP; c, purified $M_r = 100,000$ protein of 82-40 LP; d, whole cell preparation of 84-86; e, second water wash from 84-84; f, purified $M_r = 100,000$ S-protein of 84-86; g, whole cell preparation of 82-40 LP3; h, second and third water washes from 82-40 LP3; i, purified $M_r = 127,000$ S-protein of 82-40 LP3; j, whole cell preparation of 84-112; k, second water wash from 84-112; l, purified $M_r = 149,000$ S-protein of 84-112. Migration of marker proteins of known molecular weight are shown at left.

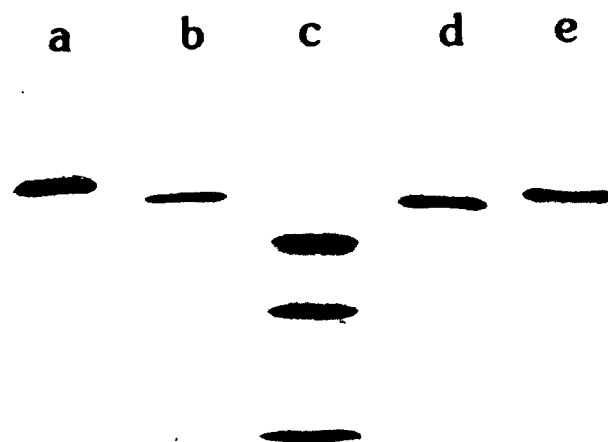


FIG. 3. Isoelectric focusing of *C. fetus* proteins. Lanes are: a, $M_r = 100,000$ S-protein of 82-40 LP (4.12); b, $M_r = 100,000$ S-protein of 84-86 (4.25); c, isoelectric standards from top, amyloglycosidase (3.55), trypsin inhibitor (4.55), β -lactoglobulin A (5.13), and bovine carbonic anhydrase B (5.85); d, $M_r = 127,000$ S-protein of 82-40 LP3 (4.21); e, $M_r = 149,000$ S-protein of 84-112 (4.20). The pI of unknown proteins was calculated by using an experient formula of pI versus distance, $y = 3.2162 + 0.1272x$; $R = 0.9995$. The difference between given and calculated pI values for standard proteins was <0.01 .

antigen [a] described by Winter *et al.* (12). These four proteins are characterized by the absence of His, Cys, and Pro and by their relative (23) hydrophobicity (37.3–38.5%). A comparison of the amino-terminal amino acid sequences of three of these proteins indicated that this region is relatively hydrophobic containing 12 hydrophobic and only 3 charged amino acids

within the first 19 residues (Fig. 4). This region is also highly conserved. Thus, these structural and composition data strongly indicate the relatedness of these proteins and demonstrate that the primary structures are homologous at the amino-terminal.

The on line search for similar amino acid sequences identified one partial match (56.3% over 16 residues) with amino acids 396 to 411 of the fusion (F) glycoprotein of human respiratory syncytial virus (24). The significance of this match is not clear.

Immunological Characterization—Results are summarized in Table II. Normal rabbit serum showed reciprocal ELISA titers of no greater than 100 against the 8 antigens used in this study. Antiserum to 82-40 HP whole cell served in this study as a purity control for *C. fetus* S-proteins, while 82-40 HP whole cells and second wash were used to evaluate speci-

ficity of the antiserum to the purified $M_r = 100,000$ protein of LP, as the HP strain differs from the parent strain (LP) by lacking only high molecular weight S-proteins (9). The purity of the *C. fetus* S-proteins and the specificity of the antiserum to $M_r = 100,000$ protein of LP were verified by evaluating cross-reactivity between antiserum to HP whole cells and the purified *C. fetus* S-proteins and between antiserum to the $M_r = 100,000$ protein and either HP whole cell or HP second wash antigens (Table II). The cross-reactions between LP whole cell and *C. fetus* S-proteins suggested that *C. fetus* proteins from different strains share common antigenic determinants on the surface of cells. This cross-reactivity was further demonstrated when antiserum to the $M_r = 100,000$ protein of LP showed a homologous reciprocal titer of 102,400 and heterologous reciprocal titers of 12,800 against three other *C. fetus* S-proteins.

TABLE I

Relative amino acid composition of high molecular weight proteins of *C. fetus*

Amino acid	$M_r = 100,000^a$	Mol % ^a			Antigen [a] ^f
		$M_r = 100,000^g$	$M_r = 127,000^h$	$M_r = 149,000^i$	
Lys	7.9	4.6	6.8	6.4	6.4
His	0.0	0.0	0.0	0.0	0.6
Arg	0.0	1.3	0.0	0.0	0.5
Asp	16.6	15.3	15.0	16.0	15.2
Thr	13.8	10.9	12.1	10.8	10.4
Ser	7.4	7.7	8.6	8.8	6.6
Glu	3.4	6.7	4.7	5.0	4.6
Pro	0.0	0.0	0.0	0.0	3.0
Gly	11.4	3.2	12.6	12.9	12.4
Ala	12.5	10.6	12.1	10.8	14.2
Cys	0.0	0.0	0.0	0.0	0.0
Val	7.0	7.0	7.7	6.8	7.4
Met	1.3	1.4	0.9	1.1	1.3
Ile	6.6	6.8	7.3	7.3	6.2
Leu	7.0	8.4	8.6	9.6	6.9
Tyr	1.9	3.0	1.6	1.7	1.2
Phe	3.0	3.1	1.9	2.6	3.0
Trp ^j	ND ^k	ND	ND	ND	0.3
Hydrophobic ^l residues (%)	37.4	37.3	38.5	38.2	42.3

^a Based on mean of duplicate determinations.

^b From 82-40 LP.

^c From 84-86.

^d From 82-40 LP3.

^e From 84-112.

^f From Ref. 12.

^g Not determined.

^h ND, not determined.

ⁱ Val, Met, Ile, Leu, Ala, Phe, Trp, and Pro (23).

DISCUSSION

Three *C. fetus* S-proteins have been purified and characterized in the present study. Amino-terminal sequence analysis and immunologic evaluation indicate that the S-proteins are highly related. It is possible that they represent a single protein that is processed to varying degrees at the carboxyl-terminal. Sequence of the entire proteins, which presently is ongoing in our laboratory, will help answer this question. Why various *C. fetus* strains preferentially produce or process one or another particular form requires further study.

Although isolation of S-layer proteins in most bacteria has included complicated treatment with urea, SDS, EDTA, EGTA, acid, or alkali (1), repeated extractions in distilled water were successfully used to isolate *C. fetus* S-proteins in the present study, and those of *A. vinelandii* by Bingle *et al.* (3). The mechanism of releasing S-proteins with water remains unknown. As suggested by Bingle *et al.* (25), the subunits of S-layers are held together by divalent cations, notably Ca^{2+} and Mg^{2+} , which slowly equilibrate into the distilled water allowing the subunits to disaggregate into monomers. The three *C. fetus* S-proteins have the following common characteristics that appear similar to other bacterial S-proteins; 1) they are easily extracted with distilled water, 2) they are highly acidic, 3) they have a hydrophobic amino acid composition while lacking cysteine, and 4) they have hydrophobic amino-terminal sequences.

For each of the *C. fetus* strains that possessed S-proteins, three distinct S-proteins of different molecular weight ranging from $M_r = 100,000$ to $\sim 150,000$ always appeared to be present. However, for each strain, one protein always was present in a major proportion; this property was generally stable after

1	5	10	15	20	25	30
100 kDa of 82-40 Met-leu-Asn-lys-Thr-Asp-Val-Ser-Met-Leu-Tyr-Ile-Thr-Ile-Met-Gly-Met-Ala-Ser-Glu						
127 kDa of 82-40 Met-Leu-Asn-Lys-Thr-Asp-Val-Ser-Met-Leu-Tyr-Ile-Thr-Ile-Met-Gly-Met-Ala-Ser-Glu-Lys-Asp-Pro-Asn-Lys-Tyr-Trp-Leu-Asp-Tyr-Ala						
149 kDa of 84-112 Met-Leu-Asn-Lys-Thr-Asp-Val-Ser-Met-Leu-Try-Ile-Thr-Ile-Met-Gly-Met-Ala-Ser						
(Gln)			(Thr)			
(Pro)						

FIG. 4. The amino-terminal sequence of *C. fetus* high molecular weight surface proteins determined with 400–500 pmol of protein, 23–42 cycles each performed once, quantitative HPLC (see "Experimental Procedures"), 94.2%–94.6% repetitive yield.

TABLE II

ELISA titer of rabbit antiserum to purified *C. fetus* $M_r = 100,000$ protein against *C. fetus* antigens

Titer is defined as highest dilution showing optical density greater than 0.100.

<i>C. fetus</i> antigens	Reciprocal titer for rabbit antibody in ELISA			
	Normal serum	Anti- $M_r = 100,000$ serum	Anti-82-40 LP serum	Anti-82-40 HP serum
82-40 LP cells	<100	3,200	25,600	25,600
82-40 LP second wash	<100	51,200	102,400	6,400
82-40 HP cells	<100	<100	12,800	102,400
82-40 HP second wash	<100	<100	12,800	25,600
$M_r = 100,000$ S-protein of 82-40 LP	<100	102,400	51,200	<100
$M_r = 100,000$ S-protein of 84-86	<100	12,800	51,200	<100
$M_r = 127,000$ S-protein of 82-40 LP3	<100	12,800	51,200	<100
$M_r = 149,000$ S-protein of 84-112	<100	12,800	12,800	<100

repeated *in vitro* passages of the strain. The S-protein with highest molecular weight was extracted in the earlier water washes while those with lower molecular weight were extracted later. In ion exchange HPLC, the major S-protein was eluted off the column by a lower salt concentration than the minor one.

The S-proteins of *C. fetus* were thought to be glycoproteins by Myers (26) and Winter *et al.* (12), but we failed to confirm this. There are several possible reasons for this discrepancy. The earlier study did not employ HPLC methods for purification. Methods used to determine glycosylation of protein at that time included the Schiff stain and colorimetric methods. No special steps were designed in the Schiff stain used by Myers to elute interfering SDS from the gel and this may have caused a false positive stain based on a simple acid-base interaction (18). Complete elimination of carbohydrate components in protein preparations is a prerequisite when using colorimetric methods to determine glycosylation because minor contamination by lipopolysaccharides and polysaccharides may cause a falsely positive result. Two independent methods were used in the present study to evaluate the purity of our S-protein preparations. Silver stain is 10 to 50 times more sensitive (17) than the Coomassie Blue stain used by Winter *et al.* (12), and immunodetection in ELISA can reveal minor contaminants that are not stained by silver. We failed to find any evidence of glycosylation in our protein preparations by using Schiff stain or deglycosylation with TFMS, and we conclude that the S-proteins of *C. fetus* are not glycoproteins.

Immunological studies by ELISA clearly show antigenic similarities of the four *C. fetus* S-proteins from different strains which support the hypothesis that these S-proteins of *C. fetus* could be used as the basis for vaccines against *C. fetus* infections.

Acknowledgments—We acknowledge Bruce Dunn, William Krugel, and Denise Swann for technical assistance.

REFERENCES

- Sleytr, U. B., and Messner, P. (1983) *Annu. Rev. Microbiol.* **37**, 311-339
- Koval, S. F., and Murray, R. G. E. (1984) *Can. J. Biochem. Cell Biol.* **62**, 1181-1189
- Bingle, W. H., Doran, J. L., and Page, W. J. (1984) *J. Bacteriol.* **159**, 251-259
- Kay, W. W., Phipps, B. M., Ishiguro, E. F., Olafson, R. W., and Trust, T. J. (1984) *Can. J. Biochem. Cell Biol.* **62**, 1074-1077
- Smibert, R. M. (1984) In *Bergey's Manual of Systematic Bacteriology* (Krieg, N. R., and Holt, J. G., eds) Vol. 1, pp. 111-118, Williams & Wilkins, Baltimore
- Guerrant, R. L., Lajtha, R. G., Winn, W. C. Jr., and Roberts, R. B. (1978) *Am. J. Med.* **65**, 584-592
- Bokkenheuser, V. (1970) *Am. J. Epidemiol.* **91**, 400-409
- Francioli, P., Herzstein, J., Grob, J.-P., Vallotton, J.-J., Mombelli, G., and Glauser, M. P. (1985) *Arch. Intern. Med.* **145**, 289-292
- Blaser, M. J., Smith, P. F., Hopkins, J. A., Heinzer, I., and Wang, W. L. L. (1987) *J. Infect. Dis.* **155**, 696-706
- McCoy, E. C., Doyle, D., Burda, K., Corbeil, L. B., and Winter, A. J. (1975) *Infect. Immun.* **11**, 517-525
- McCoy, E. C., Wiltberger, H. A., and Winter, A. J. (1976) *Infect. Immun.* **13**, 1258-1265
- Winter, A. J., McCoy, E. C., Fulmer, C. S., Burda, K., and Bier, P. J. (1978) *Infect. Immun.* **22**, 963-971
- Myers, L. L., Berg, R. L., and Firehammer, B. D. (1970) *Am. J. Vet. Res.* **31**, 1773-1777
- Blaser, M. J., Smith, P. F., Repine, J. E., and Joiner, K. A. (1988) *J. Clin. Invest.*, in press
- Markwell, M. A. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206-210
- Ames, G. F. L. (1974) *J. Biol. Chem.* **249**, 634-644
- Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1980) *Anal. Biochem.* **105**, 361-363
- Glossmann, H., and Neville, D. M. Jr. (1971) *J. Biol. Chem.* **246**, 6339-6346
- Willoughby, E. W., and Lambert, A. (1983) *Anal. Biochem.* **130**, 353-358
- Stewart, J. R., Chaplin, M. F., and Kenny, A. J. (1984) *Biochem. J.* **221**, 919-922
- Jones, B. N. (1981) *J. Liq. Chromatogr.* **4**, 565-586
- Blaser, M. J., and Duncan, D. J. (1984) *Infect. Immun.* **44**, 292-298
- White, A., Mandler, P., Smith, E. L., Hill, R. L., and Lehman, I. R., eds (1977) *Principles of Biochemistry*, 6th Ed, McGraw-Hill Publications, Minneapolis
- Collins, P. L., Huang, Y. T., and Wertz, G. W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 7683-7687
- Bingle, W. H., Whippey, P. W., Doran, J. L., Murray, R. G. E., and Page, W. J. (1987) *J. Bacteriol.* **169**, 802-810
- Myers, L. L. (1971) *Infect. Immun.* **3**, 562-566